The Potential of Solid-State Nuclear Magnetic Resonance in Dairy Research: An Application to Cheese

ABSTRACT

Nuclear magnetic resonance spectroscopy is a noninvasive technique that has found application in a variety of research areas. Nuclear magnetic resonance has been primarily applied to solution studies, but many dairy food ingredients or products are solids. In this paper, cross-polarization magic angle spinning nuclear magnetic resonance is applied to the study of freeze-dried cheese. The ^{13}C spectrum is dominated by the solid-like protein fraction. Liquid-like fat resonances can be detected by conventional one pulse experiments. The ^{31}P cross-polarization magic angle spinning nuclear magnetic resonance can specifically monitor phosphorus-containing ingredients in the solid state. Possible applications of solid-state nuclear magnetic resonance to dairy research are discussed.

(**Key words**: nuclear magnetic resonance, cheese)

Abbreviation key: **CPMAS** = cross-polarization magic angle spinning, **CSA** = chemical shift anisotropy, **MAS** = magic angle spinning, **NMR** = nuclear magnetic resonancelane.

INTRODUCTION

Nuclear magnetic resonance (**NMR**) spectroscopy is a noninvasive technique that has found widespread application in a variety of research areas, including the study of insoluble materials for which the regular solution chemical techniques are not applicable. Conventional NMR spectra of solids consist of uninformative broad peaks that may even go undetected. One major cause of this broadening is the strong dipole-dipole interactions between nuclear spins. In solution, these interactions are averaged to zero by rapid random molecular motions but are of the order of 10 kHz for carbon in organic solids where such motions are constrained. Another source of line broadening in solids is the chemical shift anisotropy (**CSA**), i.e., the dependence of chemical shift on molecular orientation with respect to the static field. The spectra of powders consist of the superposition of all these orientation-dependent chemical shifts for each nucleus. The carbon CSA can be as high as 200 ppm. In liquids, CSA is naturally narrowed by molecular motions. In order to obtain high resolution, liquid-like NMR spectra of solid samples, various line-narrowing techniques must be used (14).

The cross-polarization magic angle spinning (**CPMAS**) experiment allows the efficient acquisition of high resolution NMR spectra for isotopically (e.g., ^{13}C and ^{15}N) or chemically (e.g., ^{31}P) dilute nuclei of a solid sample. In the CPMAS experiment, the observed magnetization of the low abundance, insensitive nuclei is enhanced by transferring magnetization from the abundant, sensitive proton nuclei under a specific double resonance condition, the Hartmann-Hahn match (14, 21). This procedure has two advantages: the experiment becomes dependent on the faster proton relaxation rather than the slower relaxation of the other nucleus, permitting more rapid measurement repetitions and faster build-up of the signal-to-noise ratio; there is also an additional direct increase in sensitivity as a result of this magnetization transfer and the detection of stronger, proton-originating magnetization. Because dipolar interactions have an angular ($1\text{-}3\cos^2\theta$) dependence (θ being the angle between an internuclear vector and the static field), they can be

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eliminated by rapid mechanical spinning of the sample at an axis that makes an angle of θ = 54.7° (magic angle) with respect to the static field. Chemical shift anisotrophy has the same angular dependence. During magic angle spinning (**MAS**), all CSA patterns collapse to their respective isotropic shifts. The end effect of MAS is a dramatic improvement in resolution (14, 21). For heterogeneous samples, MAS also averages differences in magnetic susceptibility, thus eliminating another source of line broadening and improving resolution (5, 19). Residual broadening from the strong dipolar interactions between the observed nuclei and the protons is removed by high power proton decoupling (14, 21). The CPMAS experiment relies on a strong static component of the dipolar interaction between the proton and other nucleus for polarization transfer. Hence, CPMAS is efficient for immobile, solid-like molecular species but ineffective for mobile, liquid-like species, and CPMAS can be used to distinguish between the rigid and the fluid components of a heterogeneous sample (14).

This report is a preliminary investigation of the usefulness of solid-state NMR for the study of an important dairy product, cheese, which may be viewed as a distribution of milk fat droplets and water in a protein matrix (13, 22). This report also aims to identify other areas in dairy research for which solid-state NMR may provide new information.

MATERIAL AND METHODS

The shredded Cheddar cheese sample was purchased from a local supermarket. The polyacrylamide gel electrophoresis pattern of the extracted proteins (1) revealed a typical medium-aged cheese (data not shown). In initial experiments, the sample showed phase separation while in the magnet, presumably because of the fast sample spinning (3 kHz) or the radio frequency heating during high power decoupling (25 W). In order to decrease its moisture content the sample was weighed into an equal weight of distilled, deionized water and homogenized with a Polytron ST-10 (Brinkmann, Westburg, NY) for 1 min at medium speed. The homogenized sample was then freeze-dried. The freeze-dried product was free flowing, easily handled, and contained by analysis 9% moisture, 49% total fat (3), and 38% protein by Coomassie blue dye binding with casein as the standard (4). The freeze-dried cheese sample removed all the experimental hardships without any visible effect on its integrity. The freeze-dried sodium caseinate (95% protein) was prepared as previously reported (12).

Proton-decoupled ^{13}C (75.5 MHz) and ^{31}P (121.5 MHz) NMR spectra were acquired with an MSL300 multinuclear spectrometer (Bruker Instruments, Billerica, MA) using a broad band MAS probe, the temperature of which was regulated at 25°C. The CPMAS spectra were collected with matched, 50-kHz, spin-lock contact times of 1 ms (^{13}C) or 2 ms (^{31}P). About 150 to 200 mg of sample were spun at the magic angle (54.7° vs. the static magnetic field) in a double-bearing zirconium oxide rotor with a Kel-F® cap (Bruker Instruments). Other experimental details are provided in the figure legends.

RESULTS AND DISCUSSION

The ^{13}C CPMAS spectrum of cheese (Figure 1A) that contains only the solid-like cheese components is a typical protein spectrum that closely resembles the CPMAS spectrum of purified freeze-dried casein (data not shown). The conventional one-pulse ^{13}C NMR spectrum (with high power proton decoupling) detects both the solid-like and the liquid-like sample components of the sample. For cheese, the one-pulse experiment (Figure 1B) is dominated by the milk fat, which, by virtue of its absence in the CPMAS spectrum, must be primarily in a liquid-like state. This observation is of interest because milk fat is solidified below –40°C and is totally liquified above 40°C (16). In this study, the samples were measured at just above ambient temperature and should show liquid-like character. However, by varying sample temperature, estimates of the percentage of solid fat could be obtained. A further advantage of the technique is that the ^{13}C spectra avoid water interferences often seen in proton spectra (16) and could yield more reliable estimates of the percentage of solid fat in a cheese sample. The solid-like casein, which is comparable in quantity to milk fat, is undetectable as a result of its long longitudinal relaxation that does not permit efficient signal accumulation, giving rise to

inherently poor signal strengths. This behavior is similar to that of intact oilseeds (9, 18, 20) for which the CPMAS spectrum is dominated by the solid-like proteins and polysaccharides; however, the one-pulse experiment detects only the seed lipid fraction. These NMR observations for cheese are consistent with a solid-like protein matrix in which are imbedded liquid-like milk fat droplets. The one-pulse proton NMR spectrum of cheese is also indicative of simple, small mobile molecules (such as triglycerides) but not proteins (Figure 2).

The diffusion of the liquid-like milk fat and water in cheese have been studied by proton NMR relaxation measurements (2). Data allowed an evaluation of the mean size (2.1 μm) and size distribution of milk fat droplets in cheese that was independent from microscopy and suggested restricted water diffusion within the protein matrix. Other proton NMR relaxation measurements can provide information on the arrangement and domain size of the rigid components in a heterogeneous sample (15); their usefulness in the study of dairy products remains to be evaluated. In general, NMR relaxation measurements can provide information in the range of 10 to 100 Å, thus extending the 1- to 100-μm range studied by microscopy.

In the CPMAS experiment, several distinct relaxation processes are operating, each being characterized by its own relaxation time (7). When different components of a sample (e.g., protein and lactose in whey powder) are resolved in the CPMAS spectrum of the sample, comparison of their corresponding relaxation times may distinguish whether they are inti-

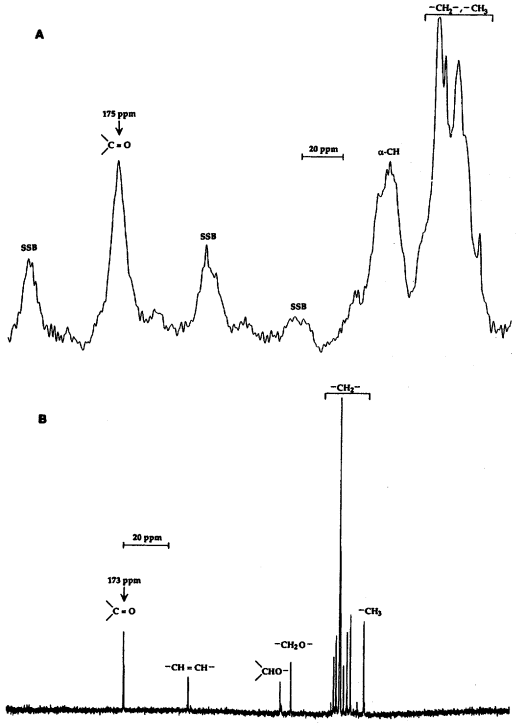

Figure 1. Use of high power, proton-decoupled ^{13}C (75.5-MHz) cross-polarization in magic angle spinning nuclear magnetic resonance (CPMAS) (A) and one-pulse (B) spectra of freeze-dried Cheddar cheese spun at 2.9 kHz. The 90° pulse widths were 5 μs (^{1}H) and 7.5 μs (^{13}C). The CPMAS spectrum (512 time-domain points zero-filled to 16 K) is the result of 3400 scans acquired with a 2-s recycle delay. For the one-pulse experiment (16 K time-domain points), 320 scans were collected with a 10-s recycle delay. In both cases, the spectral width was 25 kHz. The carbonyl peaks (protein = 175 ppm in 1A; lipid = 173 ppm in 1B) have been used to reference the spectra versus tetramethylsilane (TMS). Tentative assignments (1B) are based on published data of lipid samples (18, 20). The SSB denote spinning side bands, an experimental artifact from the insufficiently fast spinning rate that cannot average out completely the large chemical shift anisotropy of the carbonyl resonance.

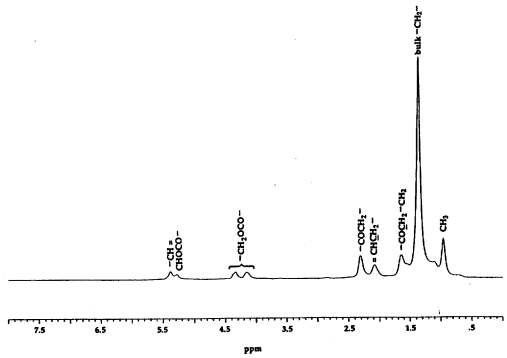

Figure 2. The proton (300-MHz) nuclear magnetic resonance (NMR) one-pulse spectrum of cheese with 2.6-kHz magic angle spinning (MAS). The spectrum was acquired using the decoupling channel of the broad band MAS probe (90° pulse width of 5 μs). Other conditions: 20-kHz spectral width, 64 scans, 10-s recycle delay, 32-K complex data points. Tentative assignments are based on published data of lipid samples (18) with the most upfield peak (methyl) referenced to .9 ppm (vs. TMS); the underlined carbon represents proton producing the assigned peak when more than two protonated carbons are shown.

mately mixed at the molecular level or they are spatially separated in distinct domains (7, 8). This result may be significant for the occurrence of chemical reactions under different processing conditions (e.g., browning of whey powder). Furthermore, the generally insoluble products of such reactions may be characterized by CPMAS NMR spectroscopy (10). Different forms of lactose can be distinguished by ^{13}C CPMAS NMR spectroscopy (6, 17). Because the lactose resonances (60 to 100 ppm) do not overlap with those of the protein, identification of lactose forms in dairy products can be achieved. Certain texture characteristics (e.g., sandiness in ice cream) are generally attributed to the form or forms of lactose present.

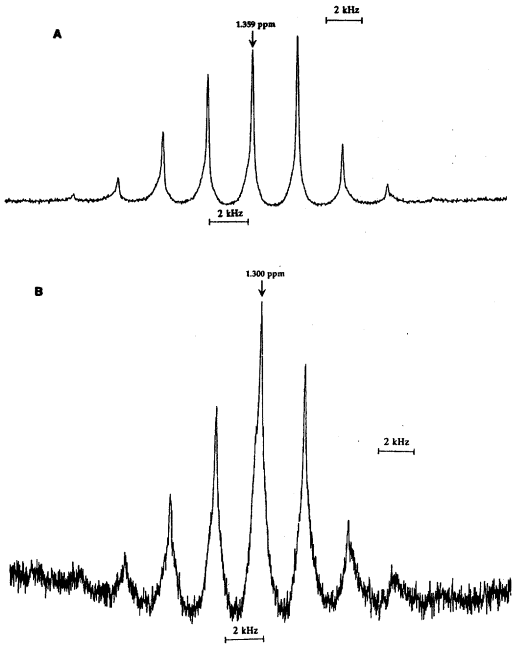

Figure 3. Use of high power proton decoupled ^{31}P (121.5 MHz) cross-polarization in magic angle spinning nuclear magnetic resonance (CPMAS) (A) and one-pulse (B) spectra of freeze-dried Cheddar cheese, both spun at 2.6 kHz. The CPMAS spectrum is the result of 1430 scans with a 5-s recycle delay; the one-pulse spectrum was obtained after 5250 scans with a 20-s delay. In both cases, the spectral width was 30 kHz. The central peak, marked with an asterisk, is the narrowed ^{31}P resonance corresponding to the isotropic chemical shift (1.36 ppm in 3A and 1.30 ppm in 3B vs. external 85% H_3PO_4). The other peaks are spinning side bands, which are an experimental artifact from the insufficiently fast spinning rate that cannot average out completely the chemical shift anisotropy of the ^{31}P resonance.

Dairy materials may also be studied by ^{31}P NMR, which is a more specific probe than either ^{13}C or ^{1}H because it detects exclusively the phosphorylated casein residues, the colloidal calcium phosphate fraction, and the milk fat phospholipids. The experimentally achieved sample spinning rates are insufficient to average the CSA of the ^{31}P resonances for a solid sample (Figure 3). Because the SSB intensities are related to the chemical shift anisotropy, such spectra may be used to characterize the sample's CSA pattern and thus obtain information on its identity and physical properties, such as molecular motion (11). Comparison of the different CSA patterns and chemical shifts (Figure 3A and B) suggest that the two experiments (CPMAS vs. one pulse) do not monitor the same sample components. The CPMAS ^{31}P NMR of dairy samples and appropriate model compounds can provide insights into the nature of micellar calcium phosphate in dairy products; results will be reported separately.

Thus, CPMAS could provide a method for evaluation of the composition of dry ingredients prior to their incorporation into products. As the field advances, even direct evaluation of products could become possible as a means of determining the solid contents and their quality.

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